THE ANTIMICROBIAL EFFICACY OF INNOVATIVE 3D TRIPLE ANTIBIOTIC PASTE-MIMIC TUBULAR SCAFFOLD AGAINST ACTINOMYCES NAESLUNDII

by

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DEDICATION

This thesis is dedicated to all the people
who sustain me in life.
To my dear parents – my father, my role model, who supported my ambition
and my pursuit of knowledge;
and my mother, always by my side with prayers and unconditional love.
To my lovely family – my husband and children, who have brought
happiness and meaning
to my life.

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First, my success and achievements come only from God. In Him I trust and unto Him I repent.

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INTRODUCTION

The field of dental biomaterials has witnessed a great revolution. Many material choices with superior aesthetic, biological, and mechanical properties have become available to restore or replace damaged and lost dental structure. However, when compared with the natural tooth structure, such materials often fail to provide the same longevity and functionality. Therefore, preserving and regenerating tooth structure is the scope of modern dentistry.

Preserving teeth during early and mixed dentition stages is essential to achieve proper function and occlusion in the permanent dentition.[1] As teeth start to erupt, their roots continue to develop to reach the final length and form. Root development continues up to three years after tooth eruption.[2] Maintaining the vitality of the dental pulp is essential to the completion of root development and to achieve apical closure. Unfortunately, pulpal inflammation and necrosis are very common in immature teeth. Dental caries and trauma are the main reasons for pulpal necrosis.[3]

Dental caries is a time-dependent multifactorial disease, which, if not treated, may lead to the penetration of bacteria and bacterial by-products into the dental pulp. Upon repeated and persistent insult, the pulp tissue undergoes chronic irreversible inflammation, ultimately leading to pulp necrosis and fibrosis.

Trauma, on the other hand, is a very common reason for pulp necrosis. It has been reported that 30 percent of children and 33 percent of adult permanent teeth are subjected to trauma.[4,5] Trauma can result in a partial or complete crushing of the apical blood

vasculature responsible for nourishing the dental pulp. Loss of blood supply eventually leads to pulp necrosis in about 1 percent to 16 percent of these traumatized teeth.[6]

Traditional root canal treatment that involves debridement, instrumentation, and obliteration of the pulp canal space is the conventional treatment modality for necrotic teeth. However, necrotic teeth with incomplete root development pose a particular challenge for this approach. Given the wide, open apex, which halts the possibility of achieving an apical seal, and the thin dentinal walls, making them more prone to fracture, performing traditional root canal treatment on necrotic immature teeth is not advisable.[7]

Apexification is the most commonly used endodontic treatment in cases of immature teeth. It relies on the use of calcium hydroxide ($Ca(OH)_2$) or mineral trioxide aggregate (MTA) to induce the formation of a mineralized barrier resulting in the closure of the apical foramen.[8] However, this approach eliminates any further possibility for complete root development (e.g., increased dentin wall thickness and ending apical formation) increasing future root fracture risks.[9,10]

Regenerative treatment, on the other hand, could promote further root development improving the long-term prognosis of immature teeth. Regenerative endodontic procedures (REPs) are biologically-based approaches that aim to restore not only the function, but also the anatomy of the damaged structures.[7] The concept of this approach is to regenerate/revitalize the necrotic pulp by utilizing the multipotent nature of stem cells derived from the periapical papilla (SCAPs). These cells can be introduced into the root canals once periapical bleeding is induced to act as a fibrin-based scaffold and develop a new pulp tissue.[7] However, the establishment of aseptic root canals

environment is a prerequisite to ensure a successful outcome of the regenerative strategy.[7,11,12]

Different bacterial species have been isolated from endodontically involved teeth.[13,14] Some of these bacteria have biofilm formation ability ranging from moderate (e.g., *Enterococcus faecalis*, *Lactobacilli spp*., and *Prevotella buccae*) to high complexity (e.g, *Actinomyces spp*, *Streptococcus mutans* and *Pseudoramibacter alactolyticus*).[15] *Actinomyces naeslundii*, a Gram-positive filamentous, rod-shaped facultative anaerobe commonly found in the oral cavity, has been reported to be associated with failed endodontic therapy.[14,16] Moreover, recent findings have identified *A. naeslundii* as the predominant bacterium in immature teeth with necrotic pulps due to trauma.[17] These findings encourage studies to test the effectiveness of traditional and new intracanal medicaments and irrigant solutions against *A. naeslundii* biofilms. According to the literature, the presence of minocycline within the so-called TAP should display an effective antimicrobial action against *A. naeslundii* biofilm (MIC = 0.23 μg/mL).[18,19]

Among the several available intracanal medicament options for regenerative endodontics, triple antibiotic paste (TAP), is the most widely used treatment. Although local application of TAP may offer advantages, such as effective root canal disinfection and a decrease in conceivable systemic complications compared with systemic antibiotic administration (e.g., antibiotic resistant strains, cytotoxicity, allergic reactions), the use of TAP has been related to significant drawbacks, including damage to stem cells and notable tooth discoloration.[4,11,20]

Electrospun polymer-based antibiotic-containing scaffolds have been shown to be an effective strategy in achieving root canal disinfection by delivering small yet effective amounts of antibiotics.[21,22]

Therefore, the aim of this study is to produce a new tubular shaped three-dimensional (3D) TAP-mimic scaffold to fit inside the root canal, and to analyze whether using the 3D-shaped antibiotic-containing scaffold will be able to completely eradicate *A. naeslundii* biofilm formed inside the dentinal tubules.

CLINICAL SIGNIFICANCE

A drug-releasing 3-D scaffold possesses favorable characteristics that could dramatically improve the outcome of the regenerative endodontic treatment. The unique nanoporous structure of the electrospun scaffold provides a more predictable skeleton to support the development of the newly regenerated pulp tissue. In addition, the slowly-released lower antibiotic dose imposes less toxicity upon the periapical stem cells (SCAPs), which will be later used in regenerating the pulp tissue.

PURPOSE OF THE STUDY

To determine the antimicrobial effect of 3D TAP-mimic scaffolds against *A. naeslundii* biofilm formed inside the dentinal tubules.

HYPOTHESES

Null Hypothesis

The null hypothesis is that the 3D TAP-mimic scaffolds will not have antimicrobial activity against *A. naeslundii* biofilm.

Alternative Hypothesis

The alternative hypothesis is that the 3D TAP-mimic scaffolds will display antimicrobial activity similar to or better than the TAP against *A. naeslundii* biofilm.

REVIEW OF LITERATURE

THEORIES AND TRADITIONAL TREATMENT MODALITIES FOR IMMATURE NECROTIC TEETH

Immature teeth with necrotic pulp have always been a challenge for endodontic treatment. In the past, different treatment approaches were suggested to overcome the problems associated with these teeth (wide, open apical foramen and thin dentinal walls). According to L.A. Friend in 1966, three basic methods existed to carry out a root canal filing in immature teeth.[23] The first method was to use a custom filling material such as gutta-percha. This material would be shaped extra-orally by the clinician and then fitted and cemented into the root canals. The problem was the difficulty of achieving a uniform seal throughout the apical third, which is usually wider than the coronal third; given the thin dentinal walls, instrumentation could not be performed to achieve a favorable conical root canal form.[23,24] The second method was to use a paste endodontic filling material. The material was injected into the canal space to achieve a uniform seal. However, the possibility of overfilling into the periapical tissue was one of the drawbacks of this approach. In the third method, advocated by Ingle 1965, a surgical intervention in the form of apicectomy is carried out simultaneously with a root canal filling. In this case an amalgam retrograde filling is properly condensed after the apicectomy to achieve an apical seal.[23,25] However, such a surgical approach is not advisable in children, who constitute the majority of immature necrotic teeth cases. In addition, apicectomy will result in a further shortening of the already short roots.[25]

Due to the limited success of the above described methods, a general interest in further root development or even the formation of an apical barrier was aroused. Nygaard-Ostby believed that the induction of bleeding by lacerating the periapical tissue will lead to the formation of a newly vascularized pulp tissue within the canal space resulting in a continued root development.[26] Moller et al. demonstrated that a successful canal debridement, which involves a complete removal of the infected pulp tissue, would create an environment that promotes the development of an apical closure without the use of medicaments.[27] The same hypothesis was promoted by McCormick et al. believing that complete pulp canal debridement is a critical factor in apexification.[28] Although most prescribed techniques involved the placement of a medicament after canal debridement, many authors believed that apical closure could still occur. Some authors also suggested that instrumentation of the pulp canal should be kept to a minimum, if at all.[29] They hypothesized that instrumentation could halt further root development by traumatizing the remnants of Hertwig's epithelial root sheath, which could potentially organize the apical mesodermal tissue into root components.[30-32]

Thus, the ultimate goal of the early work was to produce an infection-free root canal environment conducive to the formation of an apical barrier.

APEXIFICATION

Apexification is defined as "a method of inducing a calcified barrier in a root with an open apex or the continued apical development of an incompletely formed root in teeth with necrotic pulp."[33] The technique relies basically on removal of the remaining necrotic pulp tissue and cleaning of the canal, followed by filling of the root canal with a temporary filling material that would promote the formation of a calcified barrier to

achieve an apical closure of the open apex.[34] After positive radiographic and clinical assessment of the apical closure, the temporary material is replaced with a final gutta-percha filling.

In general, since the introduction of the apexification concept, many technique variations with or without the application of medicaments have been advocated for use.[24]

Calcium Hydroxide

Calcium hydroxide is one of the first used and most popular intracanal medicaments.[35] In 1964 Kaiser first introduced the use of calcium hydroxide mixed with camphorated parachlorophenol (CMCP) to induce the formation of a calcified periapical barrier.[24] Consequently, many studies have confirmed the successful outcome after using a combination of calcium hydroxide and CMCP. Calcium hydroxide was also used along with Cresatin (Premier Dental Products), shown to be less cytotoxic and to induce a lower inflammatory potential compared with CMCP.[36,37] In addition, calcium hydroxide was also mixed with saline, distilled, and sterile water in order to reduce the cytotoxic effect. Successful clinical outcomes have been reported using these combinations.[24] The mechanism by which calcium hydroxide induces the formation of an apical calcified barrier is still controversial. That is mainly because the calcium ions deposited in the apical barrier comes mainly from the bloodstream rather than the calcium hydroxide intracanal dressing.[38] Mitchell and Shankwalker have confirmed the potential of calcium hydroxide to induce the formation of heterotopic bony tissue by being implanted into the connective tissue of rats.[39] According to Holland et al., the periapical tissue reacts to calcium hydroxide in a way that is similar to the pulp tissue.[40] Calcium hydroxide produces a multilayer necrosis in the periapical area that acts as a low-grade irritation

inducing a subjacent mineralization by the attraction of calcium ions into the newly formed collagenous matrix.[24,41] It has been demonstrated that calcium hydroxide's high pH as well as its antibacterial efficacy are the main properties contributing to its ability to induce the formation of the calcified barrier. Javelet et al. demonstrated that the alkaline pH of calcium hydroxide (pH 11.8) is more conducive to the formation of an apical barrier compared with a lower pH material, such as calcium chloride (pH 4.4). Several studies have confirmed the antibacterial efficacy of calcium hydroxide attributed to the release of hydroxyl ions.[42-44] These ions are highly oxidative and can damage the bacterial cytoplasmic membrane and DNA.[24]

Calcium hydroxide can cause a localized increase in the calcium concentration, which can stimulate pyrophosphatase enzyme.[45] This enzyme can facilitate the repair process by promoting collagen synthesis.

The calcified barrier may be composed of cementum, dentin, bone or osteodentin.[32] Steiner and Van Hassel have shown that the apical calcified bridge can be histologically identified as a cementum-like material.[46] Histological analysis of this 'cap-like' bridge that extends over the root apex, revealed that it is composed of irregular dens fibrocollagenous core with calcified foci surrounded by an outer layer that is dens acellular cementum-like material. In regard to the clinical application, no agreement exists about the frequency of the calcium hydroxide dressing re-application. Authors who recommend a single application claim that calcium hydroxide plays a role only in initiating the healing reaction and that no benefit is to be gained from repeated applications.[24,47,48]

Other authors suggest that monitoring the recurrence of clinical symptoms and making a radiographic assessment of the presence of the material in the canal are the best indications for evaluating the need to replace the dressing.[49,50] However, other authors such as Abbot recommend the replacement of the dressing, because it allows for clinical monitoring of the bridge formation.[51] Abbot also believes that radiographic assessment does not yield an accurate evaluation of whether the material has been washed out nor on the progress of the barrier formation.

The time required for complete formation of the apical barrier varies according to several factors. The stage of root development, the presence of pre-treatment periapical infection, and the rate of change of calcium hydroxide are all factors that have been considered by different authors.[24]

Clinically, either a calcium hydroxide paste or a powder that is mixed with saline can be used. The creamy paste should be packed against the apical soft tissue through the canal opening. The rest of the canal length should also be filled with calcium hydroxide to maximize the antibacterial efficacy and to prevent recurrent infection throughout the extended treatment period.[52] Radiographic assessment of hard barrier formation should be conducted every three months. Evidence of material washout should also be checked to assess the need to replace the dressing. Unnecessary dressing changes should be avoided because the material's initial toxicity could delay the healing process.[53] Complete formation of the calcified barrier could take between 6 to 18 months.

Despite the high success rate of the calcium hydroxide apexification technique, it still has some major disadvantages. The prolonged treatment period, which ranges from six to eight months, involves multiple follow-up sessions making patient compliance an

essential requirement.[52] Failure of the patient to regularly appear at the follow-up appointments or loss of the temporary coronal filling between the appointments can seriously impact the outcome of this technique. Unpredictability of the apical barrier formation is another disadvantage of this approach.[54] In addition, calcium hydroxide has been shown to weaken dentin by making it more susceptible to fracture, especially in immature teeth with thin dentinal walls.[55]

Mineral Trioxide Aggregate

Due to the previously mentioned limitations of calcium hydroxide, several other materials have been considered for the treatment of necrotic immature teeth. Mineral trioxide aggregate (MTA) is a material that has been considered to replace traditional calcium hydroxide treatment. MTA was first introduced in 1993 and approved by the FDA in 1998.[24] MTA is endodontic cement that consists of fine hydrophilic particles of tricalcium silicate, tricalcium oxide, and silicate oxide. It is a biocompatible material with low solubility. After setting, the material has a high pH (12.5), which imposes some antimicrobial effect.[56]

In 1999 Shabahang et al. tested the material's ability to produce an apical barrier in immature teeth of a dog model. They concluded that MTA can successfully produce a consistent apical barrier.[57] Several clinical studies have also reported successful results using MTA.[54]

Given the previously mentioned shortcomings of the traditional multiple-visit apexification technique, a one-visit apexification that involves the use of an apical plug was suggested. The idea behind this approach is to produce an apical stop that allows direct condensation of a permanent root filling material, such as gutta-percha, eliminating

the prolonged treatment time associated with the traditional technique. MTA has been successfully used as an apical plug material. In addition to expedited treatment, the use of MTA allows for the immediate placement of a bonded core inside the root canal, thus reinforcing the canal system and decreasing the risk of fracture. MTA is placed into the apical 3 mm to 4 mm of the canal, while the rest of the canal is filled with a permanent root filling material. A bonded resin restoration is then placed to reinforce the restored tooth.[52]

Apical closure with this technique is more predictable than with calcium hydroxide; however, similar to calcium hydroxide, this technique only treats the issue of the open apex and does not result in further root development.[54]

TISSUE REGENERATION

The recent evolution in material science has greatly improved and expanded treatment options in terms of replacing damaged or lost dental structures. Nevertheless, these synthetic materials usually possess chemical, biological, and physical characteristics different from the host tissue.[58] Mostly, these artificial substitutes only provide a structural rather than a physiologically functional replacement.[1] Therefore, the long-term prognosis of traditional restorative approaches is often questionable.

The ultimate goal of any restorative treatment is to restore the tissue back to its original physiological and functional state.[59] Although unsuccessful, the early attempts to regenerate pulp tissue go back to the 1960s and 1970s.[59] Direct pulp capping, pulpotomy, and root canal revascularization are some of the earliest regenerative endodontic approaches in use since the 1970s.[60] However, the lack of isolation and

characterization strategies of the stem cells has resulted in limited success, particularly in pulp tissue regeneration.

The emergence of modern tissue engineering technology has encouraged extensive research and advancements in the field of regenerative endodontics.[59] Tissue engineering is a discipline that integrates the fundamentals of engineering, physics, chemistry and biology to develop materials and approaches that enable the regeneration of defective or lost tissues.[58,61]

The concept of endodontic tissue engineering relies on the interplay of three major components, namely, progenitor stem cells capable of differentiation to different cell types; growth factors that signal the proliferation and differentiation of the cells, and a three-dimensional scaffold that can support and provide structural integrity of the regenerated tissue.[62-64]

REGENERATIVE ENDODONTIC PROCEDURES

According to the current *Glossary of Endodontic Terms* published by the American Association of Endodontists, regenerative endodontics is defined as "biologically-based procedures designed to physiologically replace damaged tooth structures, including dentin and root structures, as well as cells of the pulp-dentin complex." [65]

In the most recent clinical considerations for a regenerative procedure, the American Association of Endodontists stated three goals of regenerative endodontic treatment for necrotic immature teeth: primarily, resolution of symptoms and evidence of bone healing; secondarily, root development in the form of increased thickness and length, and thirdly, positive evidence of vital pulp tissue growth.[66,67]

There have been several clinically successful published case reports. However, there are no adequately consistent results to support a specific regenerative protocol.[66] Based on the disinfection technique, two distinct pulp revascularization methods have been described in the literature.[68] In the first technique, calcium hydroxide is used as intracanal medicament, while triple antibiotic paste is used in the other.

Regardless of the medicament used, the AAE has published a general treatment protocol consisting of three stages.[69,70] After a thorough case evaluation and selection, the first stage involves chemical root canal disinfection using sodium hypochlorite. The AAE recommends using 1.5-percent NaOCl, however; higher concentrations up to 6 percent have been used in successful clinical case reports.[4,71] After initial irrigation, a therapeutic root canal dressing consisting of either calcium hydroxide or antibiotic paste is placed inside the cleaned empty canal. The purpose of this step is to treat any persistent infection as well as complete disinfection of the root canal to provide a favorable environment for pulp tissue regeneration.[70] In the second stage, which is typically performed in a separate appointment after 1 week to 4 weeks, the dressing is removed, and the canal is gently flushed with 17-percent EDTA. Then, the periapical tissue is lacerated using a sterile hand file to induce bleeding into the canal space. The aim of this step is to form a blood clot inside the root canal that will act as a natural scaffold for the recruited stem cells to regenerate the pulp tissue. The blood clot is also believed to help deliver SCAPs into the pulp canal space. Moreover, the disintegration of the platelets, which are a major component of the blood clot, leads to the release of growth factors essential for stem cell proliferation and differentiation. In the final stage, the bleeding is stopped and the canal is sealed with MTA; then a final restoration is carried out. A three-month interval follow-up

should be maintained to assess an indication of successful outcomes. Absence of clinical symptoms as well as radiographic evidence of bone healing and root development can take up to 2 years to be achieved.[66]

Published case reports document a successful outcome for a regenerative endodontic approach in the form of increased root length and width.[7] A greater survival rate for teeth treated with regenerative endodontic procedures (100 percent) has been noted compared with teeth treated with a traditional MTA apexification approach (77 percent).[9]

Despite these promising results, the biological outcome of the regenerative treatment approach is rather unpredictable.[70] Bone healing and root development does not necessarily confirm the regeneration of the pulp dentin complex within the root canals. In fact, histological examination of the tissue formed inside the root canals of teeth treated with regenerative procedures reveals the apposition of a cementum-like tissue, which is responsible for the canal narrowing as well as for the length increasing.[72] Additionally, in-growth of a connective tissue similar to periodontal ligament along with a bone-like tissue was identified inside the root canals.[73] These findings suggest the current endodontic regenerative protocols need careful consideration and review. The unpredictability of the results could be related to many factors. The disinfection technique is one of the most influential factors affecting the outcome of this approach.[1] There is a compelling level of evidence indicating that both irrigants and intracanal medicaments can greatly affect the survivability of the stem cells.[11,74,75]

ROOT CANAL DISINFECTION

One important factor in the success of regenerative endodontic treatments is the establishment of a high level of root canal disinfection. Regrettably, periapical abscesses and periodontitis are highly associated with necrotic immature teeth.[7] In immature teeth, bacteria are more likely to penetrate deeply into the tissue rendering bacterial disinfection even more challenging.[76,77] This step is usually accomplished using a combination of chemical irrigation and intra-canal medicaments. The current operative protocols vary considerably regarding the types and concentrations of the chemicals used. However, the majority of these protocols rely on initial root canal disinfection using sodium hypochlorite (NaOCl) or chlorhexidine (CHX) and EDTA followed by the application of intra-canal medicaments such as either calcium hydroxide ($Ca(OH)_2$) or antibiotic paste (DAP or TAP).[66,78,79]

Sodium hypochlorite

Sodium hypochlorite is one the most popular irrigants in endodontics. In addition to its antiseptic effect, NaOCl can also dissolve organic tissues including necrotic pulp remnants.[80] It has been used in variable concentrations ranging between 0.5 percent and 5.25 percent.[68] At high concentrations, sodium hypochlorite is found to be toxic to stem cells and can hamper their attachment to the dentinal surface.[74,81] Trevino et al. have shown that full-strength NaOCl is cytotoxic to stem cells and prevent their attachment to the dentinal surface of the root canals.[74] Sodium hypochlorite cytotoxicity is proportional to its concentration.[68] Martin et al. postulates that a concentration of 1.5-percent NaOCl is more favorable for the survival of stem cells as compared with a 3-percent NaOCl

concentration.[82] Generally, normal saline should be used to flush sodium hypochlorite out of the root canal to reduce its residual toxic effect on the stem cells.

Chlorhexidine

Chlorhexidine is known to have antimicrobial effects on gram positive bacteria and candida. At concentrations as low as 0.12 percent, CHX is known to be bacteriostatic, while it is bactericidal at higher concentrations. A concentration of 2 percent is the most commonly recommended. The prolonged antimicrobial effect is the main advantage of CHX. In fact, it is adsorbed to the dentinal surface allowing for an extended release that continues up to twelve weeks. However, unlike NaOCl, CHX cannot dissolve organic tissue, which is considered a major disadvantage of CHX.[68] Moreover, CHX is found to be cytotoxic to stem cells.[74] It is recommended to use normal saline between CHX and NaOCl in order to reduce the toxic effect of CHX precipitate.[83] Based on these reasons, the use of CHX in regenerative endodontics should be avoided.[66]

EDTA

Ethylenediamine tetra-acetic acid (EDTA) is a chelating agent that can strip inorganic components from the smear layer by binding divalent cations. Although it is not an antiseptic agent, EDTA can act synergistically with other irrigants (such as NaOCl) by increasing wettability of the dentin surface and removal of the smear layer. More importantly, EDTA is believed to stimulate the proliferation of stem cells by facilitating the release of growth factors as a result of its chelating effect. Additionally,

Trevino et al. found that using EDTA (17 percent) before irrigants increases the survival of stem cells.[74]

Antibiotics

A wide variety of antibiotics and antibiotic mixtures have been used in endodontic regenerative procedures. The use of a double antibiotic paste consisting of metronidazole and ciprofloxacin was reported in the first successful regenerative case report.[94] Subsequently, a triple antibiotic paste consisting of metronidazole, ciprofloxacin, and minocycline became more popular after many successful case reports.[84-86] However, minocycline was found to result in teeth discoloration.[20,87] Different triple antibiotic combinations have been tested; however, minocycline containing combinations are found to achieve the best results, especially in providing increased root thickness.[10] In fact, triple antibiotic paste containing minocycline has the ability to diffuse deeply in root dentin resulting in better disinfection of the deeper layers.[88,89] Despite its proven antibacterial efficacy, TAP is found to be cytotoxic for the stem cells, which is a major drawback for its use in regenerative endodontics.[11,72,90] Ruparel et al. have shown that the widely used creamy paste (1000 mg/mL) of a triple antibiotic mixture is toxic to SCAP.[11] Althumairy et al. postulated that the toxicity of TAP depends on the concentration. According to Ruparel et al., concentrations between 0.01 mg/mL to 0.1 mg/mL promote SCAP survival.[11] However, such low concentrations result in a watery mixture that cannot be retained inside the root canals, thus posing a clinical difficulty for using such as low concentration.[70] Therefore, it would be beneficial to use a biocompatible scaffold to deliver the antibiotic at lower concentrations.[70]

Several studies have proven the antibacterial efficacy of drug delivering scaffolds through controlled release of the incorporated antibiotic.[91, 92] Recent studies using a novel electrospun polymer-based bioactive scaffold suggest that the scaffold can release small yet effective antibiotic concentrations, which should have much lower detrimental effects on the survival of SCAP.[22, 93] In fact, in our current study an antibiotic concentration of 35 wt% was used to synthesize the scaffold yielding a total amount of less than 10 mg of TAP per scaffold, which represents only 1 percent of the total amount of antibiotic in the currently used triple antibiotic paste (1000 mg/mL).

Scaffolds

In order for stem cells to migrate and proliferate within the empty canal space, there must be a structural unit that can support this new growth.[62] A scaffold is a three-dimensional extra-cellular matrix mimicking temporary microstructure that can support and regulate cell proliferation, differentiation, and function.[94,95] Ideally, a scaffold should possess certain biomechanical properties that favor and support new cellular growth. Essential characteristics for a successful scaffold are biocompatibility; capability of seeding the stem cells and the delivery of growth factors; and bioactivity to facilitate cell adhesion and angiogenesis.[70] Mechanically, a scaffold should be rigid enough to sustain *in-vivo* stress.[58] Micro and nano-porosity is critical for cell seeding and transfusion of nutrients.[96,97] High porosity favors cellularity; however, it reduces mechanical strength.[98,99] Controllable biodegradability is one of the most critical properties of the scaffold. The scaffold material should degrade at a rate compatible with the growth of new host tissue.[100] Moreover, the biodegradation products should be non-toxic and easily eliminated.[101]

Traditionally in regenerative endodontics, the most used scaffold is a blood clot, formed by laceration of the apical tissue of a cleaned pulp canal in an immature tooth with necrotic pulp. This natural scaffold provides an environment conducive to the growth of recruited stem cells (mostly SCAPs) within the emptied root canal.[26] However, the unpredictability of the results as well as uncertainty regarding the type of regenerated tissue has nourished the search for an alternative scaffold.[72,102]

Generally, there are three basic types of scaffolds:

Natural – collagen and glycosaminoglycan, and platelet rich plasma (PRP).

Synthetic – poly-L-lactic acid (PLLA), poly-glycolic acid (PGA), and their copolymers, poly-lactic-co-glycolic acid (PLGA).

Mineral scaffold – hydroxyapatite and calcium phosphate.

Several studies report successful results using natural scaffolds such as collagen and PRP scaffolds in regenerative endodontics; nevertheless, positive histological indications regarding the nature of the newly formed tissue have not been established.[70,72]

Synthetic polymer scaffolds such as PLLA, PGA, and PLGA are commonly used in tissue engineering. Using different manufacturing techniques, these polymers can be shaped into micro or nanoporous three-dimensional structures. Such scaffolds have many favorable properties that enhance their potential role in the advancement of tissue regeneration. Great surface area, enhanced cellular adherence, the ability to deliver controlled levels of antimicrobial substances and growth factors, and controllable biodegradability are the most important properties of these synthetic scaffolds.[1]

Electrospun scaffolds

The technology of electrospinning has been recently used to fabricate nanofibrous medicated scaffolds for tissue regeneration in dentistry with promising initial results.[103-105] The nanotechnology allows for controlling fiber diameter and morphology to produce customized 3D scaffolds that fulfill the mechanical and structural demand for their use in regenerative endodontics. In addition to providing an extracellular matrix mimicking the skeleton for cell integration and proliferation, these scaffolds can be used to deliver different antibiotics and growth factors essential for infection eradication and stem cell differentiation, respectively. The most important advantage of such scaffolds is their ability to release low and controllable amounts of antibiotics with sustainable antibacterial efficacy without jeopardizing the survivability of the stem cells.[21,22,104,106]

The FDA-approved polydioxanone (PDS) synthetic polymer was used to synthesize nanofibrous scaffolds for regenerative endodontics in several recent studies.[12,21,22,103,106] PDS elicits a minimal inflammatory response compared with other synthetic polymers such as Dexon and Vicryl.[107] In addition, it has high mechanical strength and a slow degradation rate, which are suitable properties to sustain the functional demand for use as an endodontic scaffold.[107]

The incorporation of different drugs and growth factors into electrospun scaffolds is the focus of many ongoing research studies. In this study, we incorporated a triple antibiotic mixture (35 wt% ciprofloxacin, 35 wt% metronidazole, and 35 wt% minocycline) into the PDS nanofibrous scaffold to investigate the antibacterial efficacy of a novel 3D tubular-shaped drug delivery system.

ANTIMICROBIAL ASSAY

The complete eradication of infection in the root canal and the surrounding dentinal tubules is fundamental for the success of regenerative endodontics. Hence, the efficacy of a new intracanal medicament must be verified using an experimental tool that has the ability to assess these results with a high level of accuracy.[108] In chronic infections, bacteria penetrate deep into dentinal tubules and form a bacterial biofilm, which if not completely eradicated, can cause recurrent infection and thus failure of the endodontic therapy. Unfortunately, scanning electron microscopy can only be used to verify and measure the penetration of bacteria within the dentinal tubules and cannot give quantitative information about bacterial viability.[108]

Confocal Laser Scanning Microscopy (CLSM)

CLSM is an imaging technology that enables the capturing in-depth images with high resolution.[109] The technique relies on an optical sectioning feature that allows for depth selectivity. Three-dimensional reconstruction of the thin (0.5 μm to 1.5 μm) consecutive sections using computer software results in the production of a topological three-dimensional image of the specimen.

The use of CLSM in biofilm research started in the 1990s.[110] The increased focus on this technique in biofilm studies is attributable to the ability of CLSM to obtain a series of high-quality images of hydrated living sections with simple non-invasive preparation.[110,111] Currently, there are over 150 published papers that have used CLSM for oral biofilm analysis.[111] Similar to traditional light microscopy, CLSM specimen visualization is accomplished through the stimulation of fluorescent light emission using a low-power laser beam.[111,112] However, CLSM relies on the technology of spatial

filtering to eliminate out-of-focus light to produce images of well-defined sections.[112] Using CLSM is especially beneficial for the purpose of our current study. CLSM uses certain dyes to yield information about the viability of the bacterial biofilm that may have grown deeply inside the dentinal tubules. Thus, we are able to report more accurate results regarding the antibacterial efficacy of our antibiotic-containing scaffold, not only on the surface, but also in the deeper layers of the dentin specimen.

Fluorescence is the most beneficial imaging mode in biological CLSM. Fluorescent probe technology offers a high level of sensitivity with the ability to monitor specific dynamic processes and cellular integrity.[112] The LIVE/DEAD BacLight bacterial viability kit is used to gather quantitative data regarding bacterial viability. The kit is composed of two dyes: SYTO 9 and propidium iodide. The former has the ability to penetrate most bacterial membranes, while the latter penetrates only damaged cell membranes.[113] Therefore, when these dyes are applied together, viable cells with intact membranes will yield green fluorescence, while cells with damaged membranes will yield red fluorescence.[114] After *in situ* reconstruction of the images, bacterial quantification is performed using a digital image analysis tool.

MATERIALS AND METHODS

ELECTROSPUN 3D SCAFFOLDS SYNTHESIS

Polydioxanone (PDS II/ PDS, PDSII, Ethicon) suture wires were cut into small pieces and immersed in dichloromethane solution (Sigma, Aldrich, St. Louis, MO) for the undying process. After 48 h, 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma Aldrich) solution was added to the fibers at a 1:10 weight ratio in order to prepare the polymer solutions. Afterwards, three antibiotics powder namely metronidazole, ciprofloxacin, and minocycline were added to the polymer solution to prepare the triple antibiotic paste (TAP)-mimic scaffolds (at 35 wt.% of each drug). Thus, a total of 210 mg (relative to the PDS 600 mg) of each drug was incorporated into the solution. Pure antibiotic-free PDS solution, was prepared to synthesize control scaffolds. After 24 h of stirring, each solution was loaded individually into plastic syringes (Becton, Dickinson and Co., Franklin Lakes, NJ) fitted with a metallic 27-gauge blunt tip needle. Then, it was electrospun using an electrospinning system (Figure 1) consisting of a high-voltage source (ES50P-10W/DAM, Gamma High-Voltage Research Inc., FL), a syringe pump (Legato 200, KD Scientific Apparatus, Holliston, MA), and a Teflon-coated collecting steel mandrel (1.5 mm ± 0.02) connected to a high-speed mechanical stirrer (BDC6015, Caframo, Wiarton, ON) (Figure 2). The processing parameters were set up as follows: flow rate 2 mL/h, the distance between the needle tip and the collecting mandrel was 18-cm, and electrical voltage between 15 and 19 kV.

The collected tubular-shaped fibrous scaffolds were then cut into multiple specimens (1 mm height ± 0.1) (Figure 3) and dried under vacuum for at least 48 h to ensure complete removal of any remaining solvent.

DENTIN SAMPLE PREPARATION

Twenty-four caries-free human canines, collected under an approved (protocol #1407656657) local Institutional Review Board protocol (Indiana University), were cleaned and stored in 0.1-percent thymol. After removal of the crown using a low-speed water-cooled rotary saw with a thin wafering blade (Isomet, Buehler, Lake Bluff, IL), the roots were horizontally sectioned at 3 mm apical to the cement-enamel junction, to obtain 1.5-mm high (± 0.1) dentin slices (Figure 4). The specimens were wet-finished with SiC papers (800 grit) until they reached a uniform 1-mm thickness. The root canals were enlarged using a round bur (2.5 mm in diameter) at low speed (300 rpm) under water-cooling. To remove the smear layer, all specimens were immersed first in 2.5-percent NaOCl for 3 minutes in an ultrasonic bath (L&R 2014 Ultrasonic Cleaning System, NJ) (Figure 5) and washed using distilled water followed by immersion in 17-percent ethylenediamine tetraacetic acid (EDTA-Inter-Med. Inc., WI) and sonication for another 3 minutes.[115] After final rinsing with distilled water, all specimens were autoclaved (121°C for 20 min).

A. NAESLUNDII – BIOFILM FORMATION IN THE DENTINAL TUBULES

Five mL of brain heart infusion broth (BHI) was inoculated with a single colony of *A. naeslundii* (ATCC 43146) from a blood agar plate containing a pure culture of the bacterium and incubated at 37ºC in 5-percent CO2 for 16 h to 18 h. All dentin specimens were randomly placed in sterile microcentrifuge tubes containing 500 µL of *A. naeslundii* suspension pipetted on top of the dentin specimens (Figure 6). The tubes were centrifuged twice at 1400 g, 2000 g, 3600 g, and 5600 g in a sequence each for 5 minutes (Figure 7).[116] The bacterial suspension was refreshed between every centrifugation cycle, and the solution that penetrated through the dentin slice was discarded. The inoculated dentin specimens were distributed into 24 well plates containing 1 mL of BHI + 1-percent sucrose (BHIS). The plates were incubated in aerobic conditions at 37°C and 5-percent CO2 for 7 days for biofilm formation. The BHI broth was replaced every other day to remove dead cells and ensure bacterial viability. After 7 days of biofilm formation, the dentin specimens were gently rinsed with sterile phosphate-buffered saline (PBS) to remove the culture medium and non-adherent bacteria. Afterwards, the infected dentin specimens (n = 24) were randomly allocated into two experimental groups: tubular-shape TAP-mimic scaffolds, and TAP solution (50 mg/mL of each of the drugs), and two negative control groups (7-day biofilm, untreated and pure PDS). Tubular-shaped scaffolds were sterilized by UV-irradiation (30 min/side) and fitted inside the infected root canal spaces of TAP-mimic and pure PDS groups (Figure 8 to Figure 10). Triple antibiotic paste was prepared into a creamy consistency by mixing 50 mg each of metronidazole, minocycline, and ciprofloxacin with 1 mL of distilled water. The prepared TAP was applied into the root canal spaces of the third group (Figure 11). The

medicaments remained for 7 days. To maintain a humid environment and prevent the TAP and TAP-mimic scaffolds from drying out, a damp cotton ball saturated with 50 µL of distilled water was placed on top of each specimen. After 7 days, 4 of the 6 specimens of each group were washed in PBS twice and prepared for CLSM live/dead staining. The other 2 specimens of each group were prepared for SEM analysis.

Scanning Electron Microscopy (SEM)

Briefly, after biofilm formation/intracanal medicament exposure, the dentin samples (2 samples × 4 groups) were carefully removed from the wells using a sterile forceps and gently washed with PBS to remove non-adherent bacteria. The specimens were split in the middle using a sterile enamel hatchet positioned perpendicular to the flat specimen's surface and pressure applied until the specimens split into two halves. Next, the specimens were fixed in 2.5-percent glutaraldehyde for 24 hours and dehydrated in increasing concentrations of alcohol solutions (10%, 25%, 50%, 75%, 90% and 100%). The samples were mounted on aluminum stubs, sputter coated with gold and imaged by SEM (Figure 12). The dentin wall surfaces of the root canals were analyzed to verify the presence of biofilm (Figure 13).

Confocal Laser Scanning Microscopy (CLSM)

For CLSM analysis, 4 specimens from each of the 4 groups were stained with the fluorescent LIVE/DEAD BacLight Bacterial viability Kit L-7012 (Molecular Probes, Eugene, OR, USA) containing SYTO 9 and propidium iodide (PI). The stained specimens were assessed with a CLSM (Leica SP2 CL5Mt, Leia Microsystems Inc. Heidelberg. Germany) using an X40 lens. The excitation emission maxima for the dyes

are approximately 480/500 nm for SYTO 9 and 490/635 nm for PI (Figures 14 and 15). Two random areas, measuring 0.9 × 0.9 mm^2 provided by a mosaic technique, of each dentin specimen were randomly selected starting from the root canal space toward the cementum (Figure 15) to be analyzed on CLSM by 3D reconstruction. Each mosaic consisted of 9 (0.3 × 0.3 mm^2) sub-areas (Figure 16). LIVE/DEAD images were analyzed and quantified using dedicated software (Imaris 7.2 software, Bitplane Inc., St. Paul, MN).

Statistical Analysis

The percentages of green live/red dead bacteria were compared for differences of dead bacterial cells using a mixed-model ANOVA, with a fixed effect for group and a random effect for sample, to account for measurements at multiple areas on each specimen. All tests were performed using a 5-percent significance level.

RESULTS

CLSM scans and 3D reconstructions of the images of the 7-day biofilm control group showed a dense penetration of *A. naeslundii* deep in the dentinal tubules (Figure 17) with a dominant green color indicating the heavy presence of viable *A. naeslundii* inside the dentinal tubules. Bacterial viability for this group was between 99.88 and 99.99%. The PDS negative control group (Figure 18) also showed a high percentage of bacterial viability ranging between 98.01 percent and 99.34 percent. The proportion of dead bacterial cells in the 3D-TAP (Figure 19) ranged from 99.1 percent to 99.94 percent which was significantly different compared to the control PDS group ($p < 0.05$). The TAP solution group showed a 100-percent reduction in bacterial viability (Figure 20). However, there was no statistically significant difference between the results of the TAP solution and the 3D-TAP groups. Table I summarizes the results of the bacterial viability of the four groups.

Similarly to CLSM, SEM verified the penetration of *A. naeslundii* into the dentinal tubules from the root canal side after centrifugation and incubation of these specimens. SEM images showed apparent infection of the dentinal tubules in the 7-day biofilm control and the PDS groups (Figure 21 and Figure 22). Both of the TAP solution 3D-TAP groups showed bacterial-free dentinal tubules (Figure 23 and Figure 24).

TABLES AND FIGURES

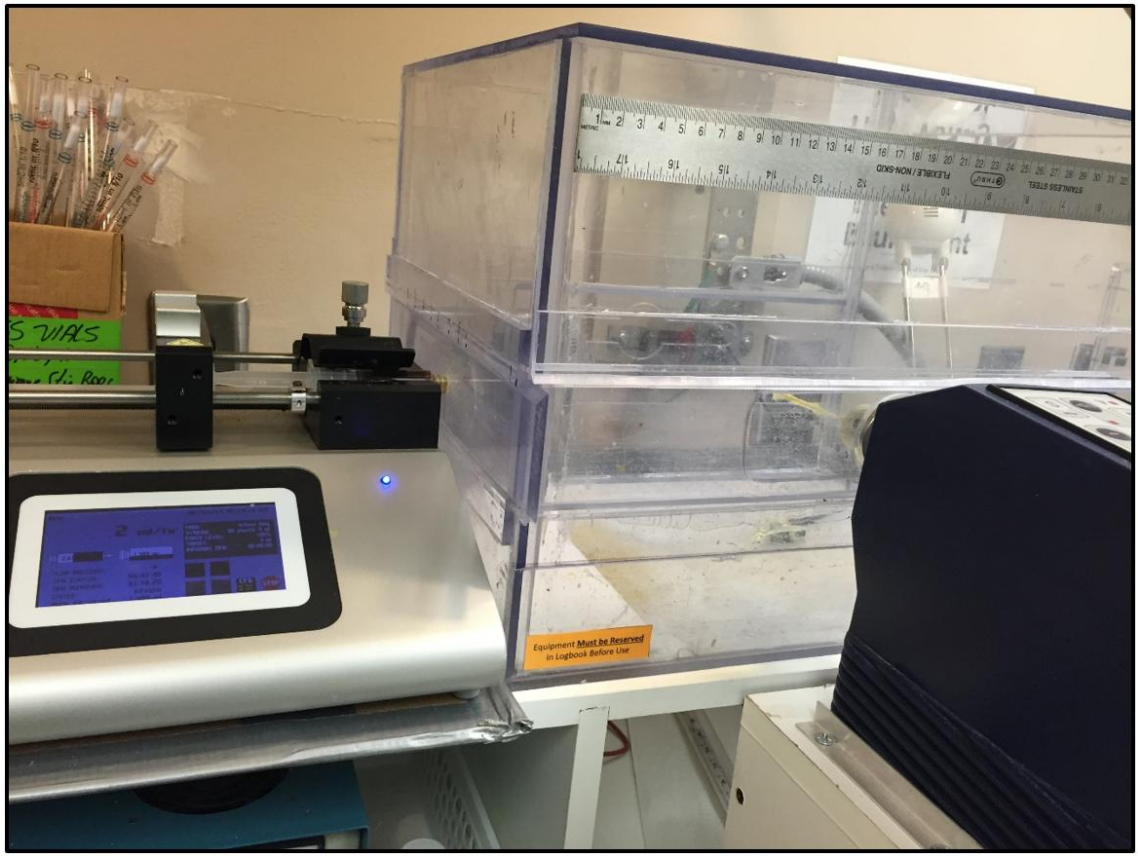

FIGURE 1. Image of electrospinning set-up used in the current study located in Dr. Bottino's laboratory.

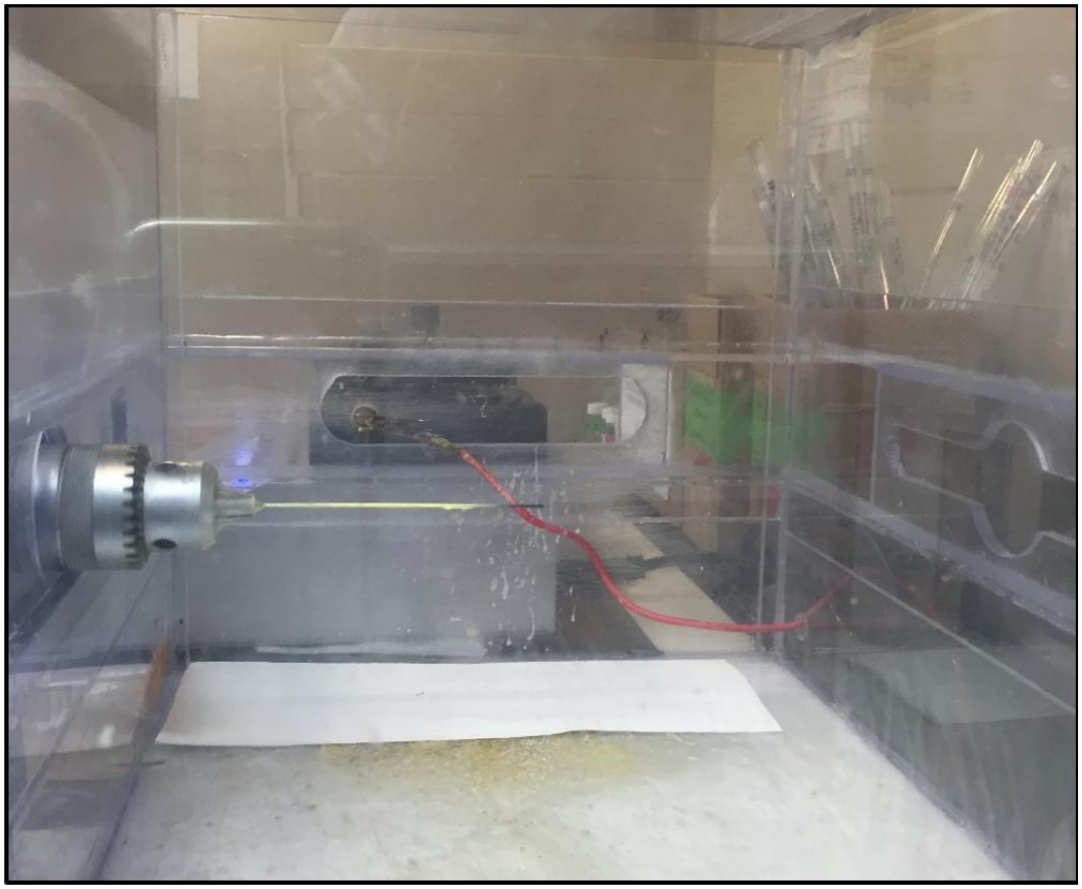

FIGURE 2. Image showing the fibrous' collection and 3D scaffold fabrication on the rotating mandrel of the electrospinning apparatus.

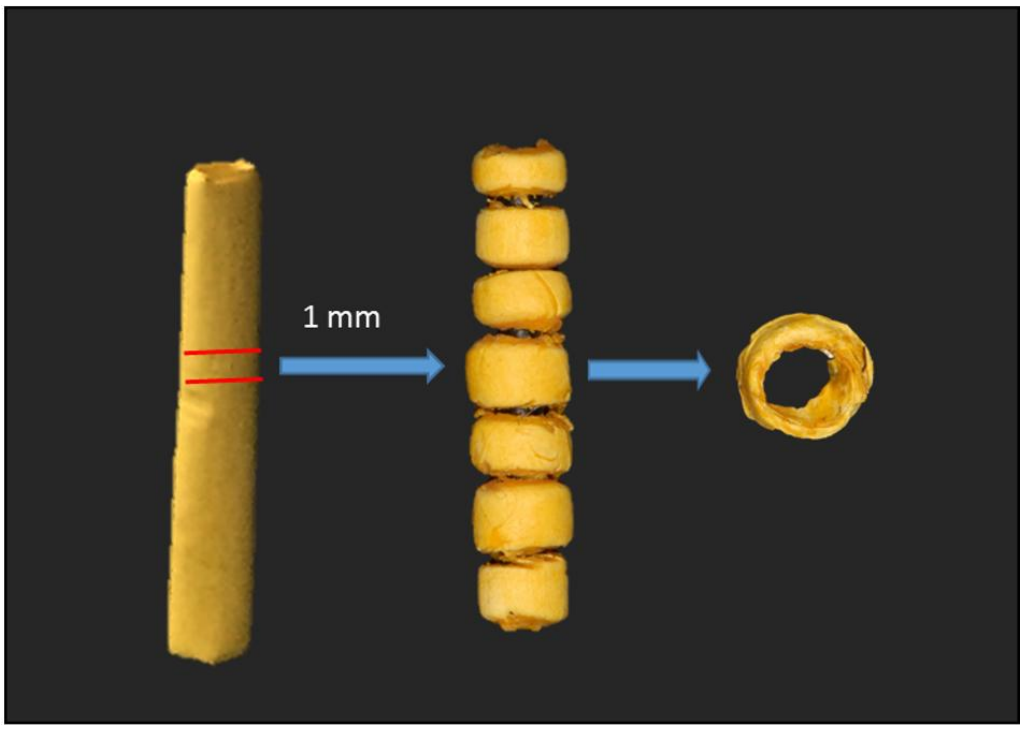

FIGURE 3. Tubular electrospun scaffold after being formed on the Teflon-coated rotating mandrel during the electrospinning process. This shows how the scaffold was cut to 1 mm height 3D-scaffolds.

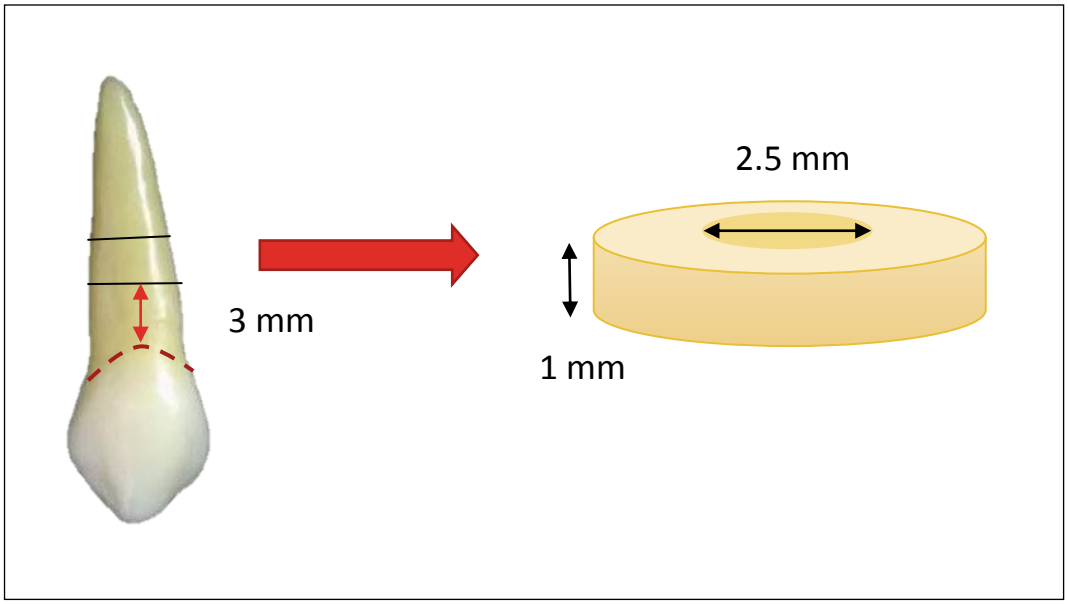

FIGURE 4. Schematic drawing showing the dimensions of the dentin specimen.

FIGURE 5. Image showing NaOCl and EDTA solutions and the ultrasonic bath used to clean the prepared specimens.

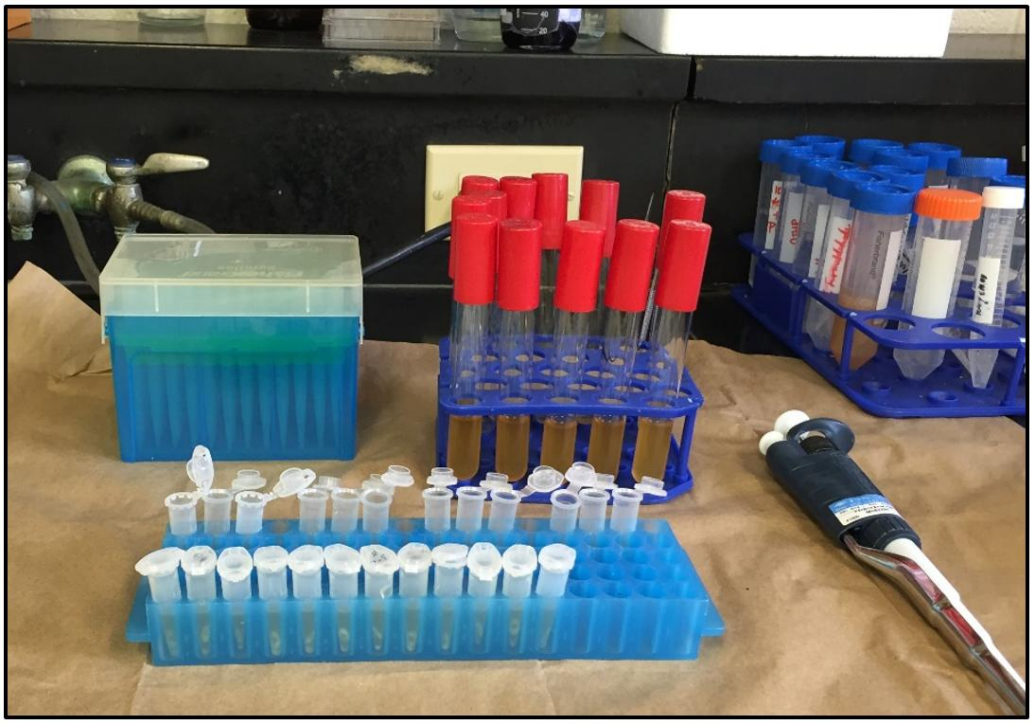

FIGURE 6. Dentin specimens placed inside microcentrifuge tubes with 500 µL of BHIS broth.

FIGURE 7. Image showing the centrifuge machine loaded with microcentrifuge tubes containing the dentin specimens.

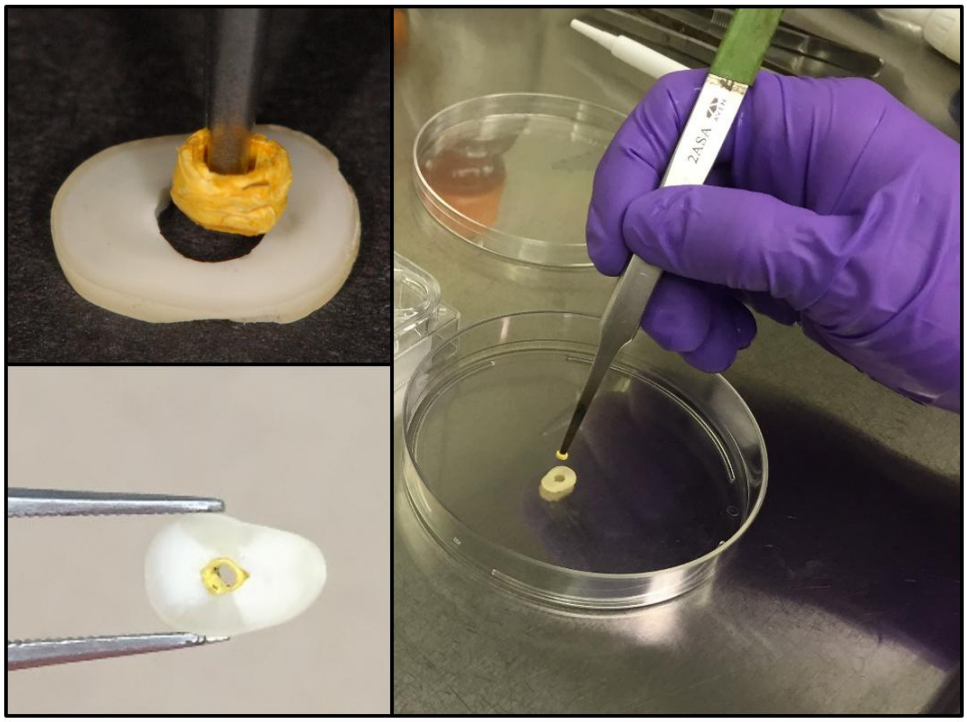

FIGURE 8. Images showing fitting the 3D scaffold inside the canal space of the dentin specimen.

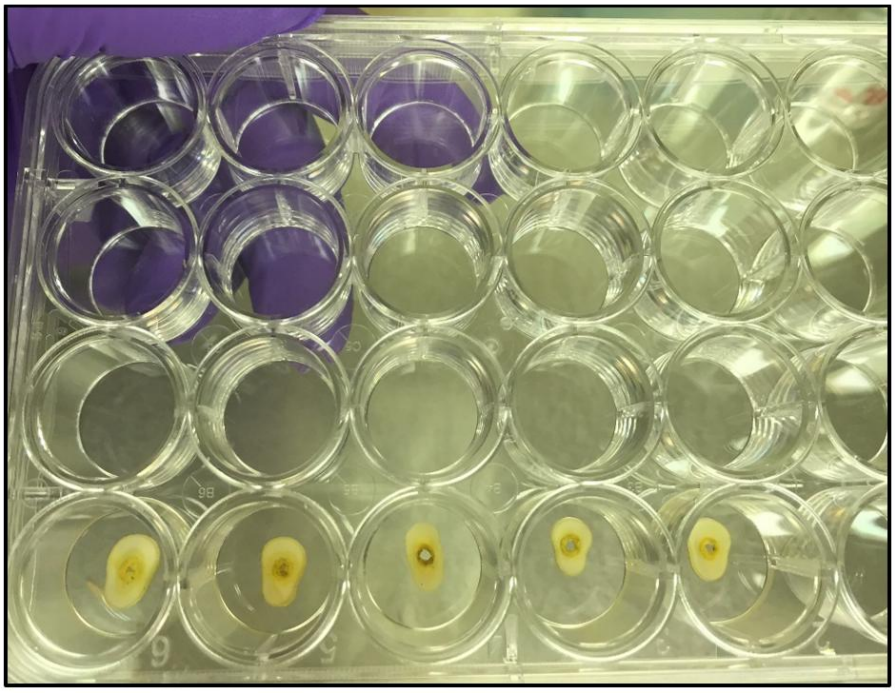

FIGURE 9. Image showing dentin specimens with 3D TAP scaffolds (experimental group) placed inside 24 well plates.

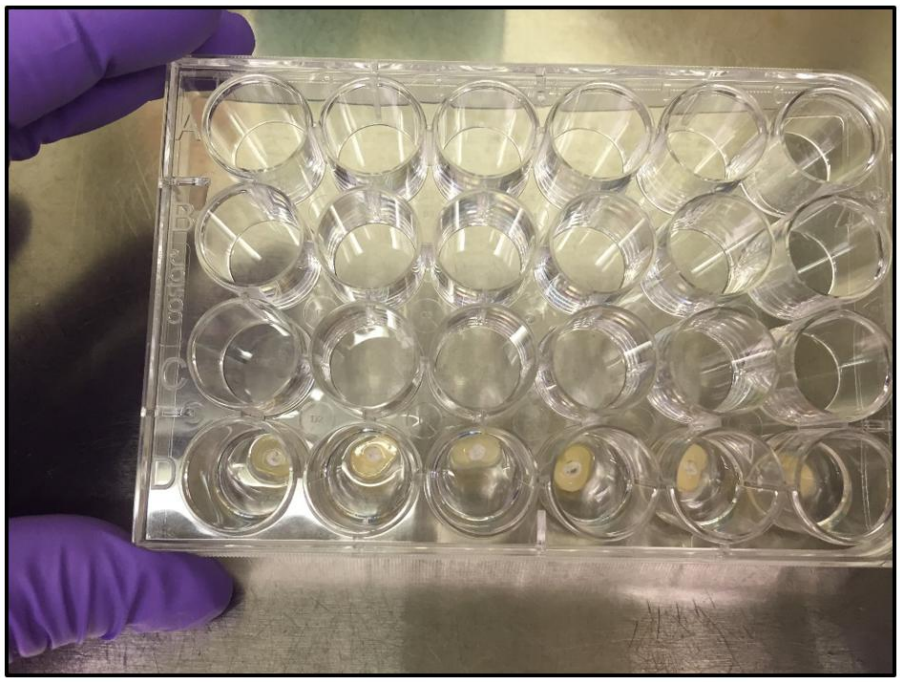

FIGURE 10. Image showing dentin specimens with 3D scaffolds (control group) placed inside 24 well plates.

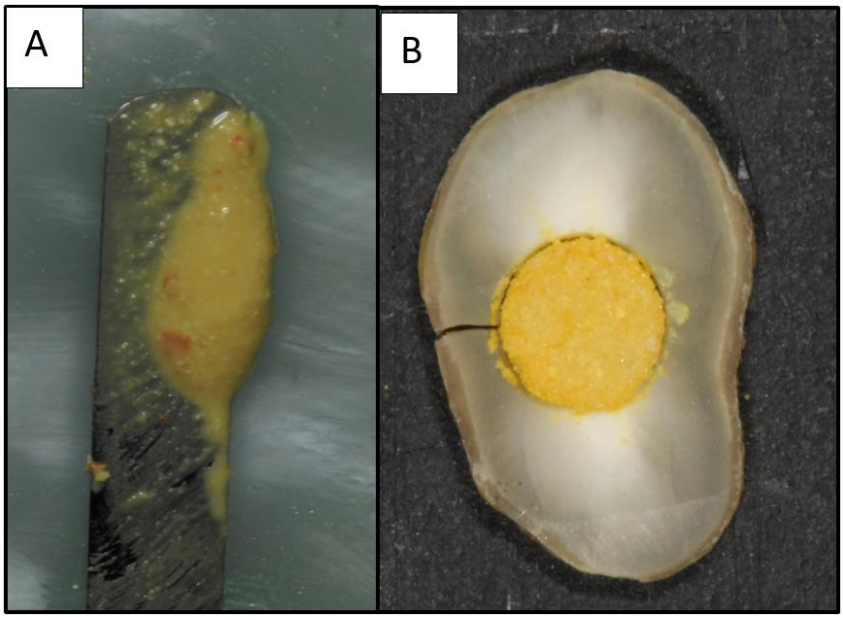

FIGURE 11. Image showing: A. The mixed TAP solution; and B. Placement inside the root canal space of a dentin slice.

FIGURE 12. Sputter coating of the specimens prior to SEM imaging.

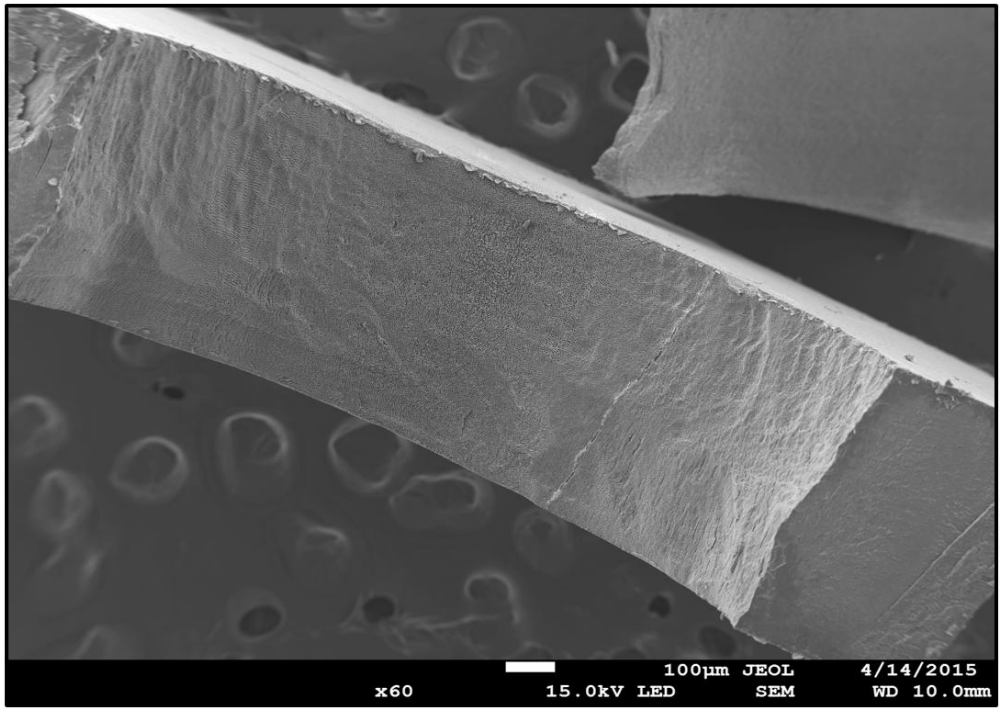

FIGURE 13. SEM image (original magnification X60) of the root canal surface which will be analyzed to verify the presence of the bacterial biofilm.

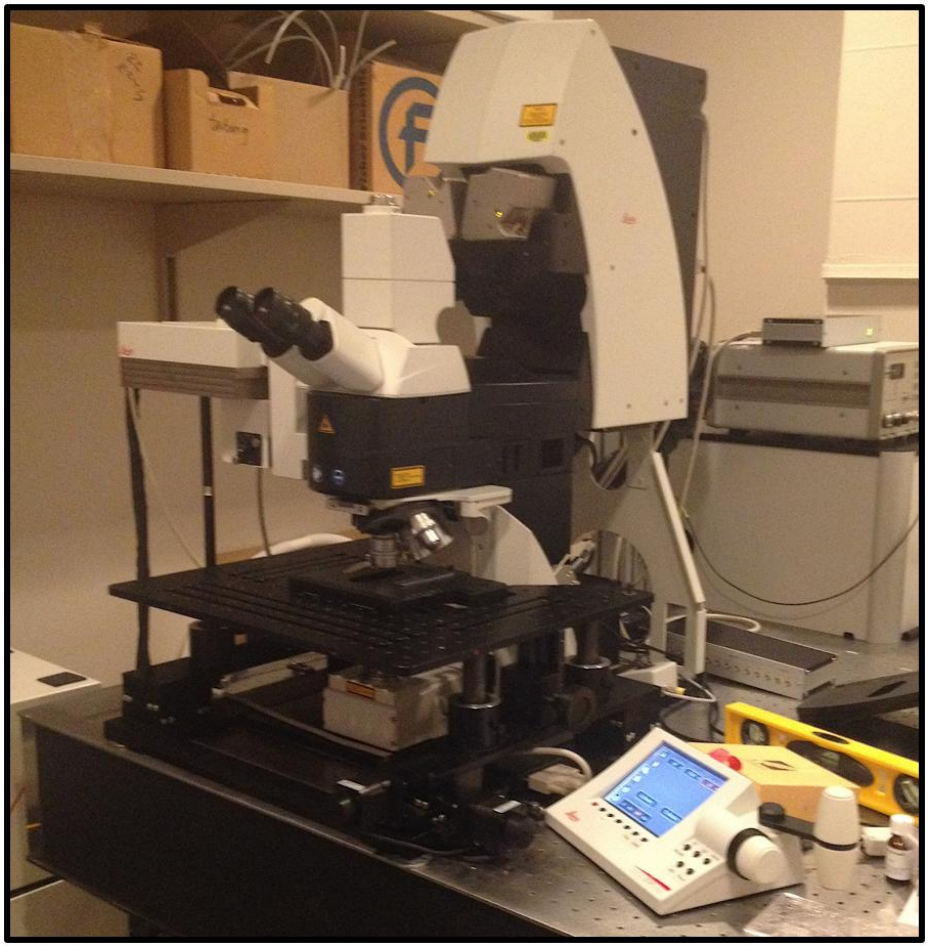

FIGURE 14. Confocal laser scanning microscope.

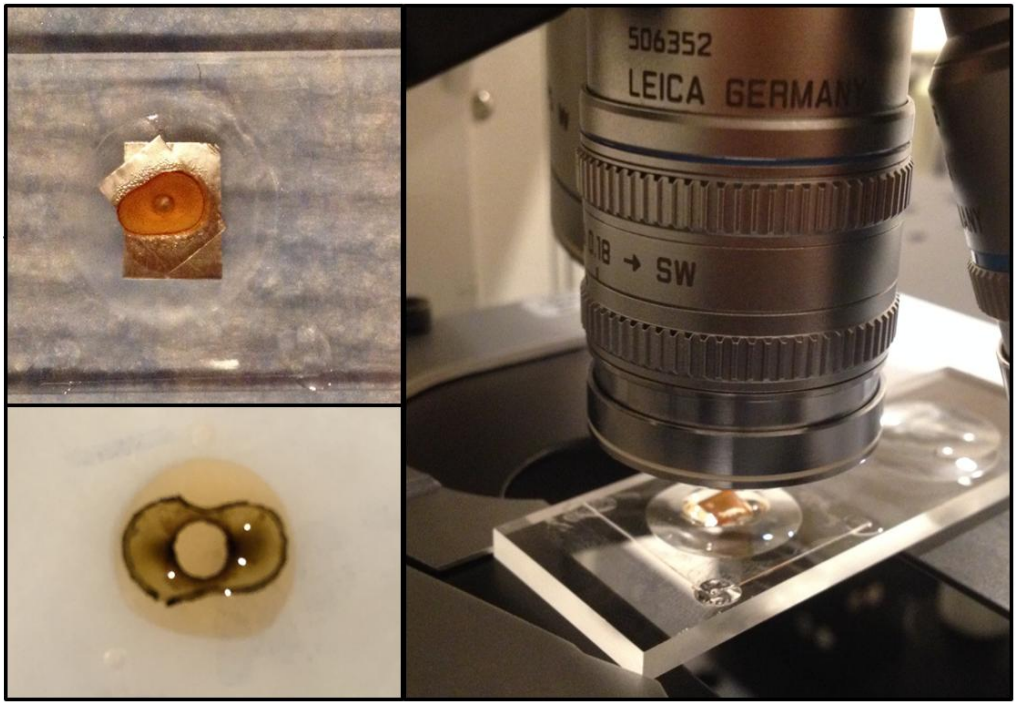

FIGURE 15. Image showing the stained dentin specimens viewed under the CLSM lens.

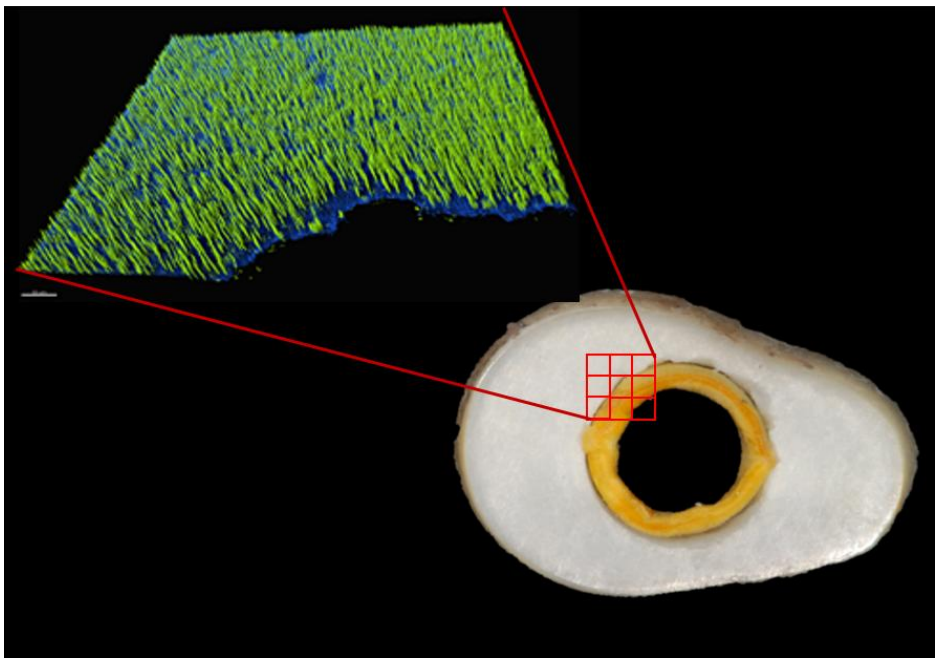

FIGURE 16. Schematic drawing showing the areas scanned by CLSM.

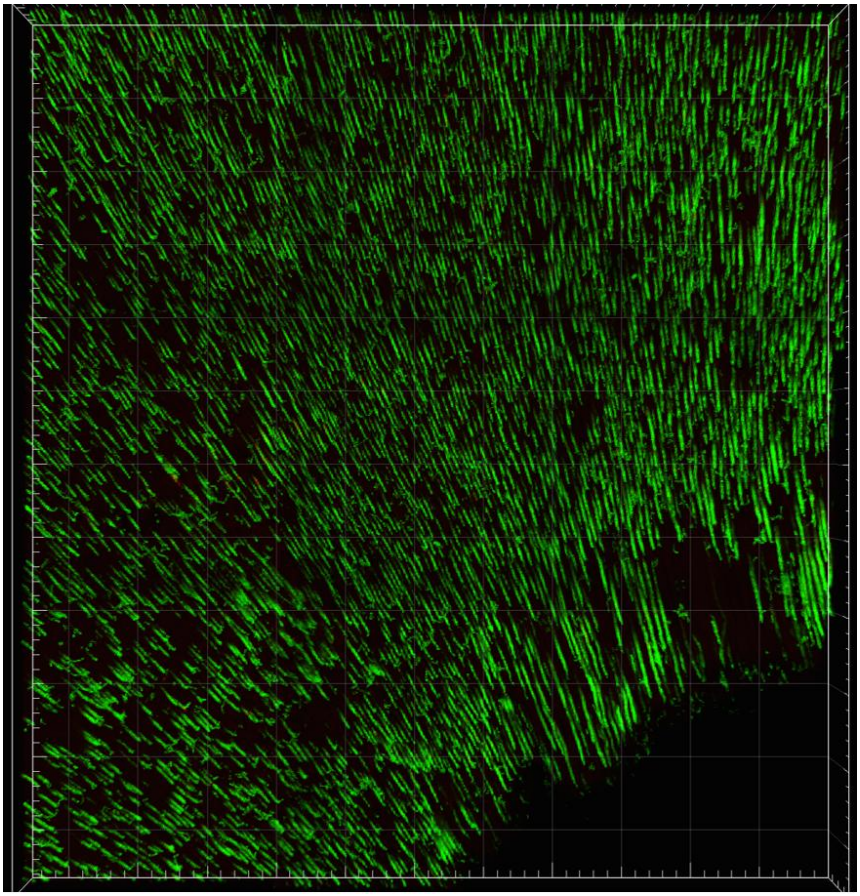

FIGURE 17. CLSM macrophotographs of 7-day *A. naeslundii* biofilm (negative control) growth inside dentinal tubules.

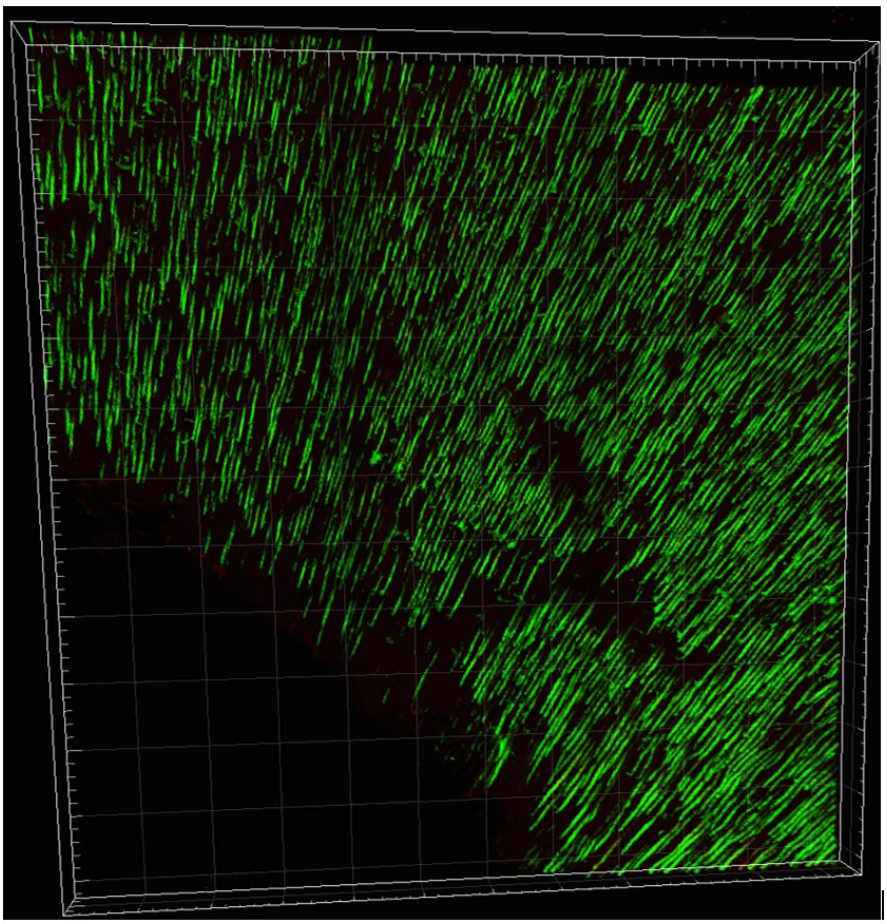

FIGURE 18. CLSM macrophotographs of infected dentin treated with pure PDS.

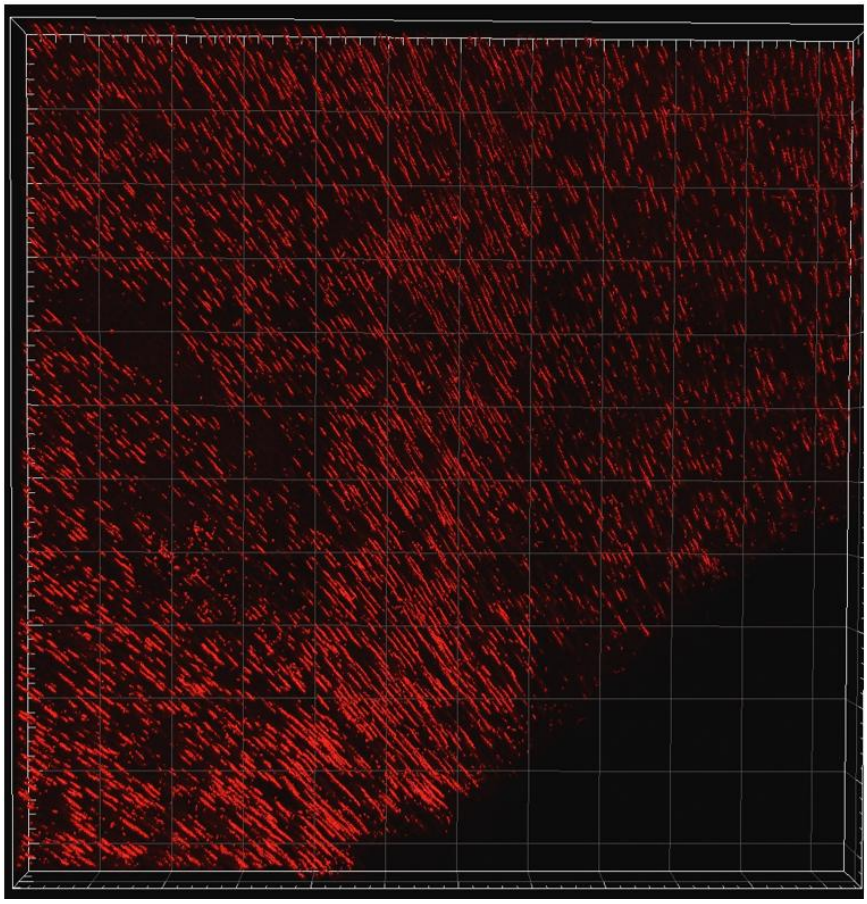

FIGURE 19. CLSM macrophotographs of infected dentin treated with 3D TAP-mimic scaffold.

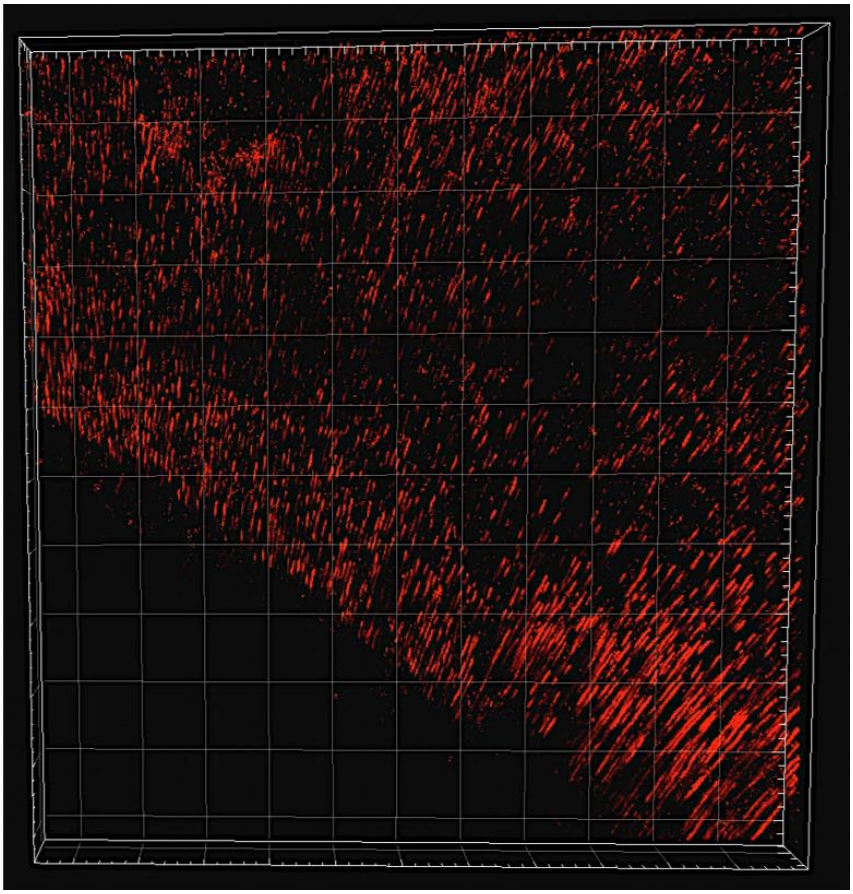

FIGURE 20. CLSM macrophotographs of infected dentin treated with TAP.

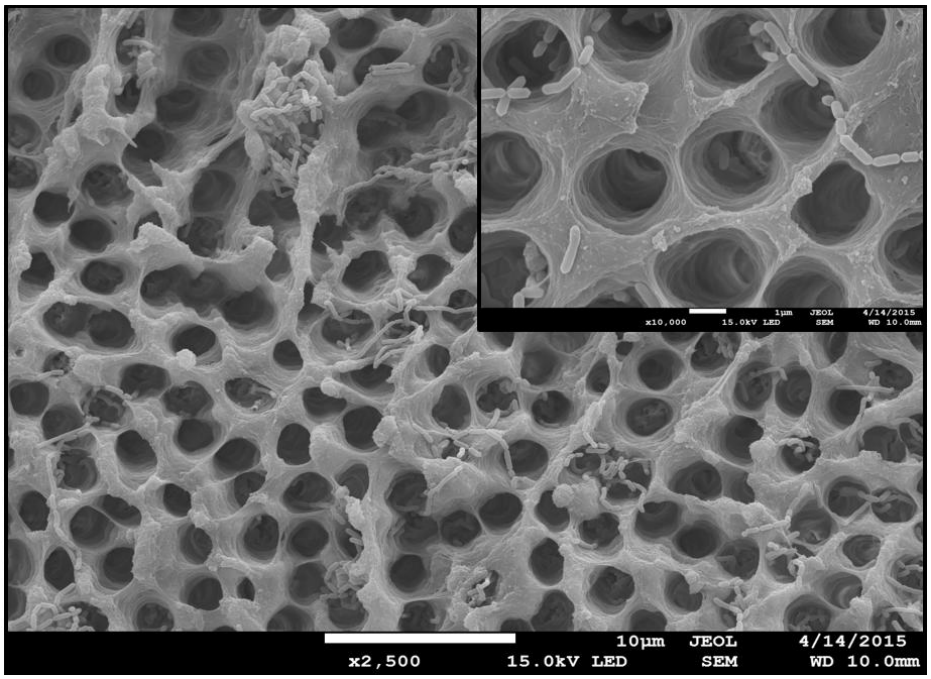

FIGURE 21. SEM images (original magnification, X2500 and X10000) of *A. naeslundii* biofilm on the dentin surface (negative control).

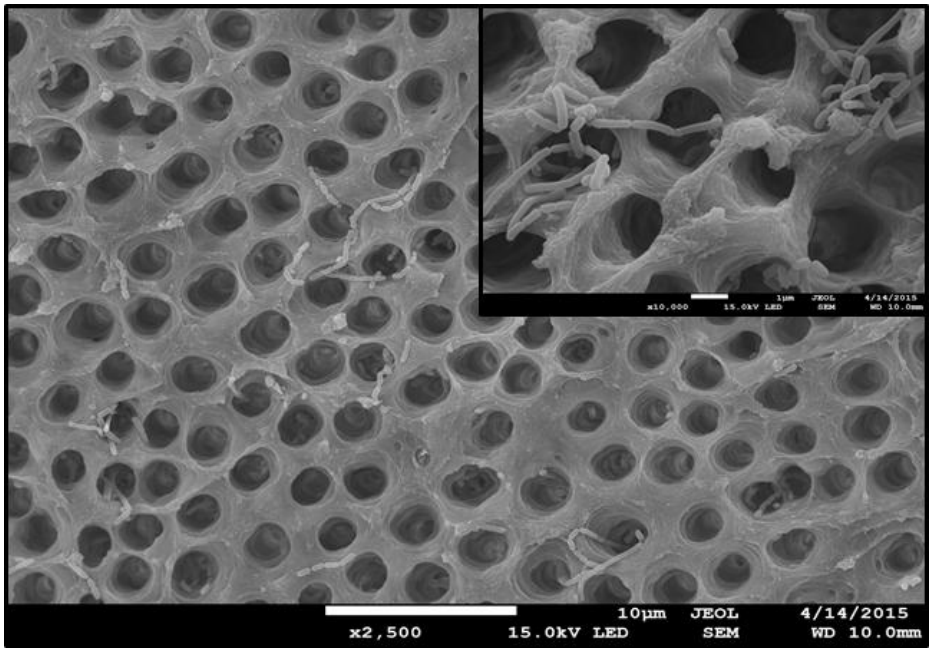

FIGURE 22. SEM images (original magnification, X2500 and X10000) of infected dentin specimen treated with pure PDS.

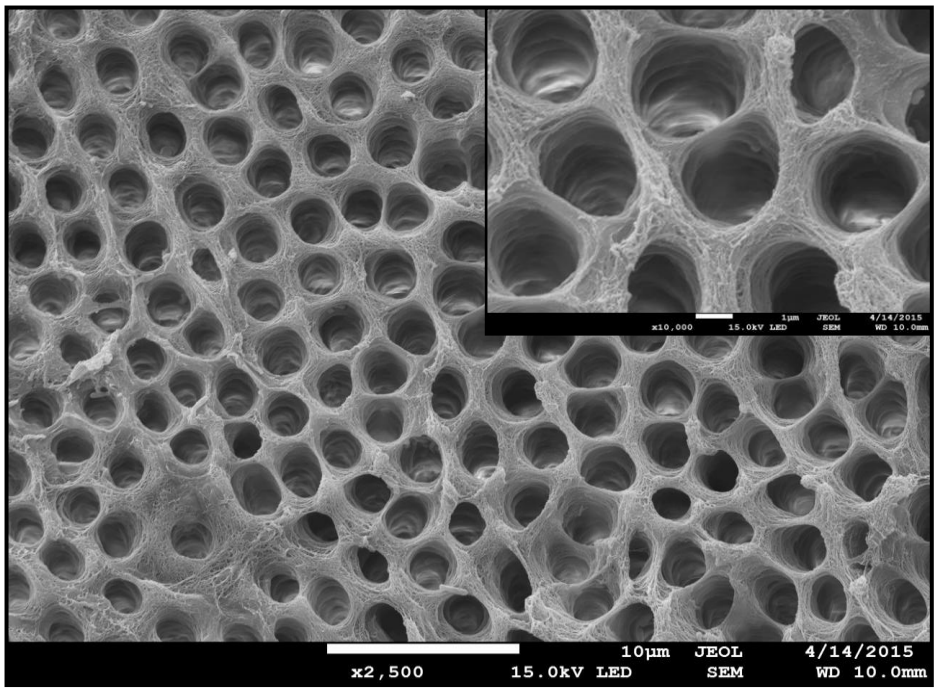

FIGURE 23. SEM images (original magnification, X2500 and X10000) of infected dentin specimen treated with 3D TAP-mimic scaffold.

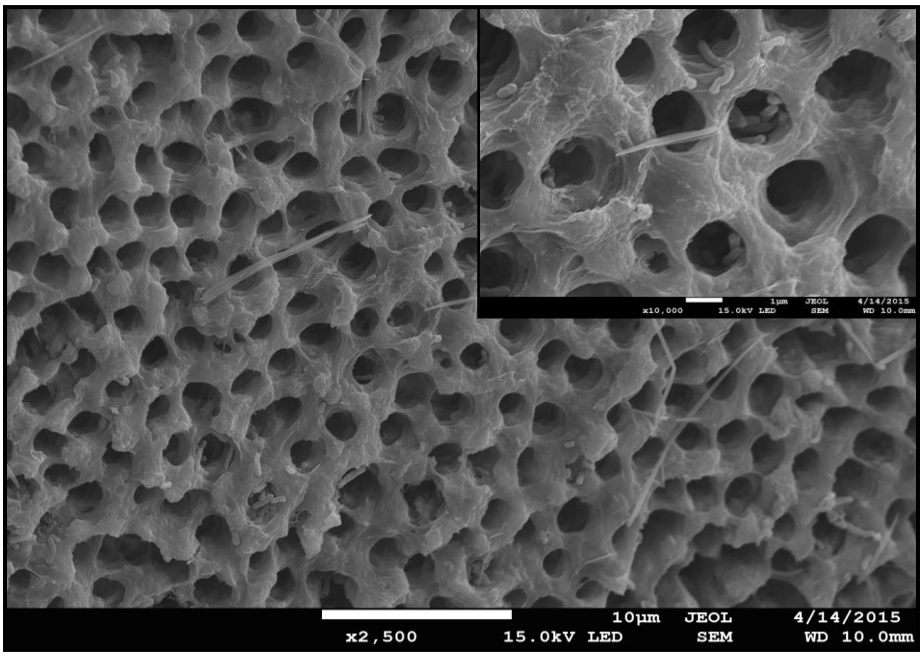

FIGURE 24. SEM images (original magnification, X2500 and X10000) of infected dentin specimen treated with TAP.

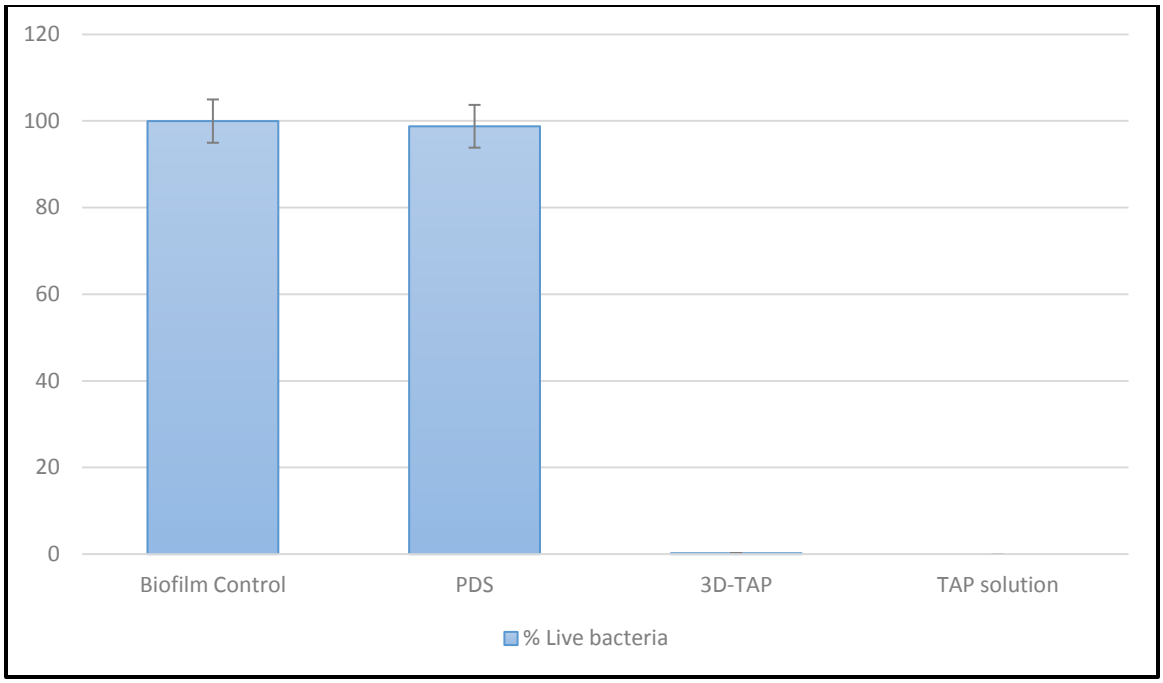

FIGURE 25. Graph representing comparison of live bacteria percentage for each of the four groups.

TABLE I

Percentage of live and dead bacterial cells based on CLSM analysis

	Groups	N Areas	N Samples	Min	Max %	Median
Dead	Control	16	4	0.01	0.12	0.03
	PDS	16	4	0.66	1.99	1.2
	3D-TAPs	16	4	99.1	99.94	99.8
	TAP	16	4	100	100	100
Live	Control	16	4	99.88	99.99	99.97
	PDS	16	4	98.01	99.34	98.77
	3D-TAPs	16	4	0.06	0.99	0.21
	TAP	16	4	0	0	0

DISCUSSION

Regenerative endodontic procedures for treating immature teeth with necrotic pulp have been increasingly utilized and investigated over the past decade.[117] Compiling successful clinical reports for cases treated with REPs have provided an evidence based strategy to replace the traditional apexification approach. In fact, recent studies suggest that the anticipated positive outcomes of REPs rationalize the use of this approach over the traditional apexification even if the achievement of the ultimate goal of pulp re-vitalization is questionable.[66]

Despite some variations, most of the current regenerative protocols emphasize infection control as an essential step in the regenerative endodontic procedures.[7,11,12] Due to the fact that mechanical debridement (instrumentation) is not recommended in cases of necrotic immature teeth, disinfection of the root canals is heavily dependent on chemical treatment. Thus, any proposed disinfectant should be able to eradicate bacteria and bacterial biofilm deep in the dentinal tubules to eliminate the chance of recurring infection. In vitro infected dentin models have been used to test the efficacy of different disinfectants used in root canal disinfection.[118-120] However, traditional broth cultured dentin specimens often fail to produce heavy infection inside the dentinal tubules.[121] In addition, quantitative comparison of live/dead bacteria between the specimens is nearly impossible due to the great variation between the specimens.[121] In this study, a centrifugation protocol was used to force the bacteria inside the dentinal tubules. In fact, this model was based on previous studies which have proven the efficacy of

centrifugation in producing multiple infected specimens with comparable amounts of bacteria [116,121] Happasalo et al. have performed extensive pilot testing on the time and force sequence used in the current centrifugation protocol.[116] It was found that shorter centrifugation cycles did not allow the bacteria to move deeper inside the tubules. A gradual increase in g force prevented bacterial accumulation on the dentin surface and allowed for deeper penetration inside the dentinal tubules. SEM and CLSM images of our seven-day biofilm control group clearly show deep penetration of *A. naeslundii* inside the dentinal tubules, and this result validates the efficacy of this protocol in producing deep bacterial infection.

The antibacterial efficacy of TAP mimic scaffold has been investigated in previous studies. Albuquerque et al. have studied *in vitro* the effects of a (TAP)- mimic polymer nanofibrous scaffold against *P. gingivalis*-infected dentin biofilm.[117] They concluded the PDS-based TAP-mimic scaffold has a significant antimicrobial efficacy against an established *P. gingivalis*-infected dentin biofilm. In a different study, the novel TAP-mimic scaffolds promoted a significant reduction in *A. naeslundii* biofilm formed on human dentin.[103] Similarly, the current study further proved the efficacy of the TAP-mimic scaffolds against *A. naeslundii* biofilm. However, the main advancement from the previous studies is the use of a more clinically relevant test model. Previous *in-vitro* studies have used a square ($4\times4\times1$ mm^3) dentin model to provide preliminary data regarding the antibacterial efficacy of the TAP-mimic scaffold. The positive initial results have promoted the development of a model that is more simulative to the proposed clinical application of this therapeutic scaffold. The nature of the root canals, as well as, the arrangement and distribution of the dentinal tubules poses a unique

anatomical challenge for the placement of the scaffolds and the action of the incorporated medicaments.

To our knowledge, this is the first study that develops a three-dimensional model to study the antimicrobial efficacy of the TAP-mimic electrospun scaffold. The tubular scaffold was fitted inside the root canal space of a dentin slice obtained from the mid-section of a human canine root. This model allows testing the efficacy of the 3D tubular scaffold against the bacterial biofilm, not only on the surface in contact with the scaffold, but also deeper inside the dentinal tubules. In fact, based on the CLSM analysis, the 3D TAP mimic scaffold has shown antibacterial effect against *A. naeslundii* biofilm that is comparable to the efficacy of the TAP (Figure 24), which is considered a gold standard for REPs. The scaffold should be in intimate contact with the dentinal wall of the root canal in order to allow efficient release of the antibiotics to act against the bacterial biofilm formed deep inside the dentinal tubules.

Collectively, the promising results of this study, in addition to similar ongoing studies in this laboratory on different bacterial species, should promote the advancement of this research to a preclinical animal research model. Such research will provide important consideration regarding the proposed clinical application of the 3D TAP-mimic scaffold. Insertion and removal techniques as well as the interaction of the TAP-mimic scaffold with the root canal environment should be addressed in future research.

SUMMARY AND CONCLUSION

Based on the present results, our null hypothesis was rejected because 3D electrospun TAP-mimic scaffold significantly reduced the percentage of viable *A. naeslundii* bacteria. The TAP mimic-scaffold showed an antimicrobial activity comparable to the TAP solution.

In fact, the TAP-mimic scaffold used in this study showed antimicrobial activity comparable to TAP, despite the lower concentration of antibiotics present in the scaffold (i.e., ~3.3 mg) as opposed to 1 g/mL in the TAP.

This scaffold has the potential to replace the currently used TAP. Moreover, it has the potential to improve the outcome of the regenerative endodontic approach by serving as an extra-cellular matrix-mimic scaffold for new tissue growth in addition to the controllable drug release.

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ABSTRACT

THE ANTIMICROBIAL EFFICACY OF INNOVATIVE 3D TRIPLE ANTIBIOTIC PASTE-MIMIC TUBULAR SCAFFOLD AGAINST ACTINOMYCES NAESLUNDII

by

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Background: Root canal disinfection is an essential requirement for the success of regenerative endodontics. Currently, the so-called triple antibiotic paste (TAP) is considered the standard of care. Notwithstanding the good antimicrobial capacity, the high concentration of TAP has shown significant toxicity to human cells, especially dental pulp stem cells. A novel drug release system, i.e., a triple antibiotic paste-mimic electrospun scaffold containing low concentrations of the antibiotics present in the TAP, has emerged as an effective and reliable alternative to fight root canal infections without potential toxic effects on dental stem cells, which are an integral part of the regenerative treatment.

Objectives: The aim of this study was to determine the antimicrobial efficacy of an innovative three-dimensional (3D) triple antibiotic paste-mimic tubular scaffold against *Actinomyces naeslundii* biofilm formed inside human root canal dentinal tubules.

Materials and methods: Pure polydioxanone (PDS) polymer solution and PDS loaded with metronidazole, ciprofloxacin and minocycline (35 wt.% of each antibiotic, 3D-TAP-mimic scaffold) were spun into 3D fibrous scaffolds. *A. naeslundii* (ATCC 43146) was centrifuged to induce biofilm formation inside human root canal dentinal tubules using a dentin slice model (1 mm thickness and 2.5 mm canal diameter). The infected dentin slices were exposed to the 3D-TAP-mimic scaffold, TAP solution (50 mg/mL of each antibiotic), and antibiotic-free PDS. Biofilm elimination was quantitatively and qualitatively analyzed by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM), respectively.

Results: A dense penetration of *A. naeslundii* biofilm was observed by CLSM throughout the dentinal tubules. 3D-TAP-mimic scaffold significantly reduced the percentage of viable bacteria compared with PDS ($p < .05$). TAP solution completely eliminated viable bacteria without differing from 3D-TAP-mimic scaffolds. SEM images showed results similar to CLSM.

Conclusion: Collectively, the proposed tubular 3D-TAP-mimic scaffold holds significant clinical potential for root canal disinfection strategy prior to regenerative endodontics.

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